A model system of the dynamic loading occurring in synovial joints: The biological effect of plowing on pristine cartilage

Correro-Shahgaldian, M Rita ; Ghayor, Chafik ; Spencer, Nicholas D ; Weber, Franz E ; Gallo, Luigi M

Abstract: Mechanical stress is listed as a main risk factor for cartilage degradation. The aim of this study was to investigate the biological response of cartilage to dynamic loading such as plowing. Cartilage strips harvested from bovine nasal septum were submitted to plowing using a cylindrical indenter, applying a constant normal force in the vertical axis and moving at constant speed in the horizontal axis. After plowing, cell viability, gene expression and glycosaminoglycan (GAG) release were measured with conventional assays. The cell-viability assay and qRT-PCR showed that plowing induces cell death and matrix metalloproteinase 3 (MMP-3) upregulation. The addition of actinomycin D, before or after plowing, confirmed that plowing was responsible for the observed MMP-3 upregulation. Even if the transcriptions of the tissue inhibitor of metalloproteinase (TIMP-1), aggrecan (Agg), collagen type I (Coll1), collagen type II (Coll2) and fibronectin (Fn) were not significantly affected by plowing, actinomycin D treatment revealed that plowing induces a strong increase in TIMP-1 and Coll1 messenger RNA content and influences the gene regulation of Agg, Coll2 and Fn. Furthermore, plowed cartilage explants exhibited enhanced GAG release. Application of hydroxamate MMP inhibitor after loading showed that plowing induces GAG release via the activation of catabolic enzymes. Plowing causes cell death of the chondrocytes closer to the surface as well as matrix damage, observed as GAG loss. Moreover, in healthy chondrocytes, plowing promotes the production and activation of catabolic enzymes like MMP-3.

DOI: https://doi.org/10.1159/000375294

A model system of the dynamic loading occurring in synovial joints: the biological effect of plowing on pristine cartilage

M. Rita Correro-Shahgaldian [1, 2], Chafik Ghayor[2], Nicholas D. Spencer[3], Franz E. Weber[2] and Luigi M. Gallo[1]

[1]Clinic for Masticatory Disorders, Removable Prosthodontics and Special Care, Center for Dental Medicine, University of Zurich, Switzerland

[2]Oral Biotechnology & Bioengineering, Division of Cranio-Maxillofacial and Oral Surgery, University Hospital Zurich, Switzerland

[3]Laboratory for Surface Science and Technology, Department of Materials, ETH Zurich, Switzerland

Address correspondence to: Franz E. Weber, Universitätsspital, Oral Biotechnology & bioengineering, Frauenklinikstrasse 24, CH-8091 Zürich, Switzerland
Phone: +41-44 255 5055
Fax: +41-44-255 4179
e-mail franz.weber@zzm.uzh.ch

Biological effect of plowing on pristine cartilage

Key words

• synovial joint

• cartilage

• plowing

• biomechanics

• biochemistry

• chondrocytes

• gene expression

• extracellular matrix

Abstract (215 words)

Mechanical stress is listed as a main risk factor for cartilage degradation. The aim of this study was to investigate the biological response of cartilage to dynamic loading such as plowing.

Cartilage strips harvested from bovine nasal septum were submitted to plowing using a cylindrical indenter, applying in the vertical axis a constant normal force and moving at constant speed in the horizontal axis. After plowing, cell viability, gene expression and glycosaminoglycan (GAG) release were measured by conventional assays.

Cell-viability assay and qRT-PCR showed that plowing induces cell death and MMP-3 up-regulation. The addition of actinomycin-D, before or after plowing, confirmed that plowing was responsible for the observed MMP-3 up-regulation. Even if the transcriptions of TIMP-1, aggrecan, collagen-type-I, collagen-type-II and of fibronectin were not significantly affected by plowing, actinomycin-D treatment revealed that plowing induces a strong increase in TIMP-1 and collagen-type-I mRNA content and influences the gene regulation of aggrecan, collagen-type-II and fibronectin. Furthermore, plowed cartilage explants exhibited enhanced GAG release. Post-loading application of GM6001, a metalloproteinase inhibitor, showed that plowing induces GAG release by activation of catabolic enzymes.

Plowing causes cell death of the chondrocytes closer to the surface, as well as matrix damage observed as GAG loss. Moreover, plowing promotes, in healthy chondrocytes, the production and activation of catabolic enzymes, such as MMP-3.

Introduction (670 words)

Over time, mechanical stress is one of the causes of cartilage degradation. In order to understand the pathomechanics of cartilage breakdown occurring in the synovial joints, the response of cartilage to mechanical loading has been extensively studied by means of several laboratory models [Kurz *et al.*, 2005]. Interestingly, it has been reported that moderate loading helps cartilage homeostasis [Dossumbekova *et al.*, 2007; Griffin *et al.*, 2005; Lane *et al.*, 2000; Torzilli *et al.*, 2010] but that injurious overload contributes to or causes cartilage degradation [Chen *et al.*, 2003; DiMicco *et al.*, 2004; Ding *et al.*, 2010; Lee *et al.*, 2005; Lin *et al.*, 2004; Patwari *et al.*, 2001; Patwari *et al.*, 2003; Sauerland and Steinmeyer, 2007; Verteramo and Seedhom, 2007]. Chen *et al.* [Chen *et al.*, 2003] have shown that continuous or intermittent uniaxial loads of varying durations and magnitudes applied to bovine articular cartilage explants cause chondrocyte death and collagen damage. DiMicco *et al.* [DiMicco *et al.*, 2004] reported that uniaxial unconfined compression of bovine articular cartilage explants causes preoteoglycan (PG) degradation and subsequent loss of glycosaminoglycans (GAGs). Moreover, uniaxial cyclic loading of cartilage explants leads to cell death, collagen damage and GAG loss. It was also shown that long-term mechanical stress causes an increase in stromelysin-1 (matrix metalloproteinase 3, MMP-3) activity [Lin *et al.*, 2004]. Others [Ding *et al.*, 2010; Fitzgerald *et al.*, 2006; Lee *et al.*, 2005; Sauerland and Steinmeyer, 2007; Verteramo and Seedhom, 2007] have reported that static compression of articular cartilage explants causes specific, time-dependent changes in chondrocyte gene expression and that cyclic uniaxial compression or shear stress regulate clusters of functionally related gene patterns. Although these models reproduce *in vitro* the mechanical stresses that cartilage experiences *in vivo* and provided insights into the biological response of such a complex tissue to mechanical injuries, their uniaxial design presents some limitations.

In a previous study, we studied the temporomandibular-joint (TMJ) disc deformations occurring during mandibular function by means of novel, three-dimensional modelling software that processes data acquired by magnetic resonance imaging (MRI) and jaw tracking (a system that allows observing mandibular movement dynamically) [Gallo 2005; Gallo *et al.*, 2006; Palla *et al.*, 2003]. These TMJ disc measurements were used to develop a rolling/plowing explant test system (RPETS) that is able to mimic the *in vivo* plowing effect resulting from the combination of compression and sliding of the mandibular condyle on the TMJ cartilage disc [Colombo *et al.*, 2011].

In the present study we report on the biological response of bovine nasal septum (BNS) cartilage to plowing by studying the chondrocyte viability, the gene-expression variation and the GAG release.

Materials and methods (1237 words)

Harvesting of cartilage explants Nasal septa of 12-month-old calves were provided by a local abattoir within 4 hours after slaughter. Under sterile conditions, control cartilage explants (20 x 30 x 2 mm) and cartilage strips (60 x 17 x 2 mm) were harvested by using a "dual-parallel-blade cartilage cutter". Control samples and cartilage strips were washed in Dulbecco's phosphate-buffered saline (D-PBS) (Invitrogen Carlsbad, CA, USA) and equilibrated overnight in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with fetal bovine serum (10%) (Sigma St. Louis, MO, USA), Hepes (4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid) buffer (10 mM) (Sigma), nonessential amino acids (0.1 mM), penicillin (100 units/mL), streptomycin (100 µg/mL) and amphotericin-B (0.25 µg/mL).

Plowing of cartilage strips Before plowing, cartilage strips were glued by their ends to plexiglas supports, by means of cyanoacrylate glue. The plowing was performed unidirectionally in DMEM for 2 hours, at 37°C in a sterile hood, and was carried out by using a not-rotating, cylindrical indenter (∅ 25 mm; stainless steel) moving in the horizontal axis with a constant speed of 10 mm/sec and simultaneously compressing the cartilage in the vertical axis by applying normal forces of 25, 50 or 100 N.

The indenter stroke length was 40 mm and the total number of plowing cycles was 500. During plowing, the control cartilage was placed, as a free-swelling explant in the medium in which the cartilage strip was located.

After plowing, cartilage sub-explants (15 x 15 x 2 mm) were collected using a blade from both the plowed strip and the free-swelling control, and subjected to analysis.

Chondrocyte viability assay Following plowing (at 25, 50 or 100 N applied normal force), cartilage sections (500 µm) were sectioned, rinsed in D-PBS and incubated for 20 min in DMEM containing 1µg/ml calcein acetoxymethyl ester (CAM) (live-cell staining; Sigma) and 1 µM ethidium homodimer (dead-cell staining; Sigma). The treated slices were thereafter transferred into fresh DMEM and fluorescence-microscopy images were acquired at 515 and 635 nm by means of an inverted fluorescence microscope (Zeiss, Axiovert 200) equipped with a digital camera.

RNA extraction and quantitative, real-time polymerase chain reaction (qRT-PCR) After plowing at 25, 50 and 100 N applied normal force, cartilage sub-explants collected from the plowed strips and from the controls were equilibrated for 2, 4 or 24 hours in DMEM at 37°C. Upon equilibration, samples were snap-frozen in liquid nitrogen and stored at -80 °C. RNA extraction was performed according to Davidson *et al*. [Davidson *et al*., 2006] with some modifications. Finely sliced cartilage sub-explants (≈ 50 mg) were placed in Eppendorf tubes and homogenized twice for 1 min in 800 µL TRIzol reagent (Invitrogen). After 5 min equilibration at room temperature, 200 µL of chloroform were added and the tubes were vigorously shaken, mixed and incubated for 2 min at room temperature. Following centrifugation at 9.5 g for 30 min at 4 °C, the obtained aqueous phases were recovered, extracted with 200 µL of chloroform and treated as previously described. The recovered supernatants were transferred into 2 mL tubes, gently mixed with 500 µL of isopropanol, incubated for 10 min at room temperature and subsequently centrifuged at 9.5 g for 40 min at 4 °C. The supernatants were discarded and the pellets resuspended in 900 µL of lysis buffer (RNeasy Mini Kit®; Qiagen GmbH, Hilden, Germany) supplemented with 90 µL β-mercatpoethanol (Sigma-Aldrich). After adding 900 µL ethanol (75 %), the RNA was purified

using a Qiagen RNeasy mini kit, while genomic DNA was digested with a DNase kit (Qiagen) according to the manufacturer's instructions. Reverse transcriptions of RNA were performed with random hexamer primers using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics AG, Rotkreuz, Switzerland), according to the manufacturer's instructions. qRT-PCR of the obtained complementary DNA (cDNA) was performed in 96-well plates by means of an iCycler Real-Time Detection System® (iQ5Bio-Rad Laboratories, Hercules, CA, USA) and the reactions were carried out using a QuantiFast™ SYBR® Green PCR kit (Qiagen). The used primers were the same as those reported in Fitzgerald *et al*. [Fitzgerald *et al.*, 2006]. They were designed for the amplification of selected target genes: the extracellular matrix proteins aggrecan (Agg), collagen-type-I (Coll1), collagen-type-II (Coll2), fibronectin (Fn), the catabolic enzyme MMP-3 and its inhibitor (the tissue inhibitor of metalloproteinase (TIMP-1)), and the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and ribosomal RNA 18S (18S-rRNA). Housekeeping genes as controls from treated samples and gene of interest from untreated control samples were run on every plate for normalization purposes. The gene regulation was calculated as a multiple, by using the comparative threshold cycle (Delta-Delta Ct) method. Thus, a gene was considered up-regulated when the multiple of expression was higher than 2 and down-regulated for values lower than 0.5.

GAG release In order to measure the GAG release following plowing, cartilage sub-explants were collected from strips plowed at 100 N applied normal force and from free-swelling controls. The samples were incubated in DMEM at 37°C during four different post-plowing equilibration durations (1, 2, 3, or 4 days). For each time point, cartilage sub-explants and 1 mL of the corresponding culture medium were collected and stored at -20 °C until a 1-9-dimethylmethylene blue (DMMB) (Sigma™) assay was performed [Barbosa *et al.*, 2003]. GAG measurement in the media was carried out according to Jeffrey *et al*. [Jeffrey and Aspden, 2007]. Briefly, 50 µL of appropriately diluted medium samples or chondroitin-sulphate-A (10-100 µg/mL) (Sigma) standard were mixed with 1 mL of DMMB solution (16 mg/L DMMB in 0.2 M guanidine hydrochloride (GuHCl), 1 g/L sodium formate and 1 mL/L

164 formic acid). The absorbance at 525 nm was immediately read in triplicate in a 96-well plate
165 using a spectrophotometer plate reader (Synergy HT multi-Mode Microplate Reader,
166 BioTek).
167 ***Inhibition experiments*** To confirm that the change of chondrocyte gene expression was
168 induced by plowing, two different experiments were performed as follows:
169 1) Cartilage strips and controls were incubated in DMEM supplemented with 30 µMol
170 transcription inhibitor actinomycin-D (Sigma™) for 2 hours before plowing. The strips were
171 then plowed for 2 hours with 100 N applied normal force. In this experiment, the effect of
172 plowing on gene expression was blocked, so that no difference with regard to controls was
173 expected if gene expression changes were due to mechanical loading.
174 2) Conversely, cartilage strips were plowed for 2 hours with 100 N applied normal force and
175 then incubated for 2 hours with the transcription inhibitor. The control cartilage was subjected
176 to the same treatment in inhibitor. In this case, we would detect only genes expressed during
177 plowing.
178 After both types of experiments, gene expression of MMP3, TIMP-1, Coll1, Agg, Fn and
179 Coll2 were determined by qRT-PCR.
180 To confirm that plowing causes GAG release by activating catabolic enzymes, experiments
181 of MMP inhibition were performed by using hydroxamate matrix metalloprotease inhibitor
182 (GM6001) (Millipore, Bedford, MA USA). Briefly, cartilage strips and free-swelling controls
183 were incubated for 4 hours before plowing in serum-free DMED supplemented with 1%
184 sodium selenite, insulin, transferrin and ethanolamine (SITE) (Sigma) and containing 10 µM
185 GM6001. After 2 hours plowing at 100 N, applied normal forces performed in serum-free
186 medium, cartilage sub-explants (three for each strip) were collected from both control
187 cartilage and plowed strips and incubated again in DMEM containing GM6001 and SITE for
188 1, 2, 3 or 4 days.
189 ***Statistical analysis*** All experiments were carried out in triplicate, each time from a different
190 nasal septum. Results are expressed as the mean ± SE. Statistical differences were
191 analyzed using two-way ANOVAs and Student's ***t*** tests.

Results (865 words)

Cell viability After plowing of cartilage strips, chondrocyte viability was studied by means of CAM and ethidium-homodimer staining (Fig.1). A layer of dead cells was detected on the surfaces of the explants. The layers varied in thickness with the magnitude of the applied normal force: they corresponded to 4.6 % ($p<0.01$), 7.3 % ($p<0.01$) and 8.7 % ($p<0.001$) of the total sample thickness for strips plowed at 25, 50 and 100 N, respectively.

Effect of plowing on cartilage gene transcription after 2, 4 and 24 hours post-plowing equilibration Upon plowing completion at 25, 50 or 100 N, cartilage explants from plowed strips and relative controls were equilibrated for 2, 4 or 24 hours, and at each time point, the effect of plowing on expression of the selected genes (MMP-3, Fn, Coll1, Coll2, Agg and TIMP-1) was measured using qRT-PCR (Fig. 2).

After 2 hours equilibration, cartilage explants subjected to 25 N plowing revealed only a slight change in the MMP-3 messenger RNA (mRNA) synthesis (1.8-fold up-regulation) and this value increased (2.3-fold) after 4 hours but it dropped to 0.9-fold after 24 hours equilibration. Furthermore, the cartilage strips subjected to 50 N plowing had 4.7-fold up-regulation of MMP-3 after 2 hours equilibration, and this value decreased over time (4.1-fold after 4 hours and 2.4-fold after 24 hours equilibration).

The up-regulation of MMP-3 for the strip subjected to 100 N plowing was stronger: 6.3-fold after 2 hours, 5-fold after 4 hours and 3.2-fold after 24 hours post-plowing equilibration.

In general, the decrease in MMP-3 expression was statistically significant, comparing the expression after 2 and 24 hours for 25 and 100 N applied normal forces, (for 25 N: $P = 0.0001$; for 50 N: $P = 0.0700$; for 100 N: $P = 0.0075$).

The plowing at the chosen applied normal forces did not influence the net gene expression of Agg, Fn, TIMP-1 and type I and II Collagens, whose levels remained at around the pre-plowing values (Fig. 2).

Transcription inhibition experiments Upon plowing completion and without inhibitor treatment, MMP-3 was 8.6-fold up-regulated (Fig. 3A) but when cartilage strips were treated with actinomycin-D for 2 hours before plowing, no MMP-3 up-regulation was observed upon

plowing completion (Fig. 3A) ($P = 0.0001$). In contrast, when the treatment with the transcription inhibitor was performed for 2 hours after plowing, MMP-3 was 7.8-fold up-regulated (Fig. 3B). This value was not different from the MMP-3 expression of plowed cartilage measured after 2 hours equilibration without actinomycin-D ($P = 0.45$) (Fig. 3B).

Besides MMP-3 gene regulation, actinomycin-D treatment of cartilage revealed that plowing also influences TIMP-1 expression. Upon plowing completion, TIMP-1 expression was 1.7-fold up regulated (Fig. 3A) and similarly, when cartilage strips were pre-incubated for 2 hours in media containing actinomycin-D and then subjected to 100 N plowing, the gene expression of TIMP-1 was 2-fold increased (Fig. 3A). Moreover, cartilage incubated in actinomycin-D for 2 hours after plowing, induced a strong (7.5-fold) TIMP-1 up-regulation (Fig. 3B) ($P = 0.0010$). Since qRT-PCR of plowed cartilage strips not treated with inhibitor revealed that TIMP-1 expression measured after 2 hours equilibration was unaffected by plowing (as shown in Fig. 3B), these results suggest that when transcription is enabled, the TIMP-1 mRNA content is reduced. As shown in Figs. 3A and B, the mRNA content of collagen-type-I was similar to that of TIMP-1, indicating that plowing also reduces the stability of collagen-type-I mRNA ($P = 0.0010$). The other studied genes, Agg, Fn and Coll2, which normally were slightly or not affected by plowing when no inhibitor was added, showed a different time course. Indeed, when incubation in actinomycin-D was performed either before or after plowing, levels of expression of Agg, Fn and Coll2 remained at around 2 fold up-regulation (Fig. 3A and B). In particular, statistical analysis revealed that the expression of Agg increased because of the treatment with actinomycin-D ($P = 0.009$ and $P = 0.007$ for treatment before and after plowing respectively). A similar behaviour was observed for Fn ($P = 0.010$ and $P = 0.017$ for treatment before and after plowing, respectively) and Coll2 ($P = 0.011$ and $P = 0.004$ for treatment before and after plowing, respectively).

Effect of plowing on GAG release To determine if post-plowing GAG release was mechanically or enzymatically induced, MMP activity was inhibited by GM6001 treatment of the cartilage explants (controls and plowed samples) before and after plowing. As shown in

Fig. 4, all samples showed increasing GAG release over time with excellent coefficients of determination (R^2 values ≥ 0.9).

GAG release from plowed samples (filled squares and filled circles), independently of the MMPs inhibitor treatment, was higher than that from the corresponding controls (empty squares and empty circles).

Additionally, the GAG release from the control samples (empty squares and empty circles), showed no statistically significant difference, but the plowed samples treated or not with MMPs inhibitor (filled squares and filled circles) showed statistically significant differences of GAG release over time (for day 1: P = 0.011; day 2: P = 0.003; day 3: P = 0.018; day 4: P = 0.012). These results suggest that GAG release is due both to a mechanical and an enzymatic component and that under plowing conditions the enzymatic component is dominant.

Discussion (1467 words)

In the present study we performed plowing of pristine cartilage strips and demonstrated that *in-vitro*, it causes cell death at the surface of the explants, changes the chondrocyte gene expression and induces GAG release by activating catabolic enzymes.

Bovine nasal cartilage was selected as model tissue because of its unique features: it is pristine (not previously subjected to any mechanical load), it has homogeneously distributed chondrocytes and together with being easily available in big quantities, it can be easily shaped. Additionally, it has been recently demonstrated that bovine nasal cartilage behaves as a biphasic material and has viscoelastic responses to dynamic forces [Colombo *et al.,* 2013].

During jaw opening/closing, the TMJ cartilage disk is mainly affected by the plowing forces that are the dominant components of the tractional force. The plowing of the disk results from the combination of an applied normal force and the sliding of the condyle.

The plowing parameters used in this study were comparable to those encountered under physiological conditions. The indenter speed was chosen after evaluation of TMJ recordings performed during rhythmic jaw opening and closing [Gallo *et al.*, 2000]. Interestingly, this

speed value can also be compared to that estimated in other joints, such as in the knee during walking [Waldman and Bryant, 1997]. The applied normal forces were chosen according to the study of Seller and Crompton, showing that 100 N corresponds to the condylar TMJ force occurring during biting [Sellers and Crompton, 2004].

In this study, we provided a model of cell death caused by plowing. We found that plowing of cartilage strips induces cell death progressively, increasing with the magnitude of applied normal force. The fact that dead cells were mainly detected at the surfaces of the explants suggests that chondrocytes located closer to the surface are more exposed to mechanical stress and therefore more vulnerable than those in the deeper zones. Furthermore, qRT-PCR revealed that MMP-3 up-regulation, similarly to the superficial cell death observed, is dependent on the magnitude of the applied mechanical stress and that it decreases over a 24 hours post-plowing equilibration period (Fig. 2). In contrast, the mRNA content for Agg, TIMP-1, Fn and for collagens type-I and type-II remained unchanged compared to the control values. As active MMP-3 digests collagens, PGs, and other ECM proteins and additionally activates the pro-forms of other MMPs and aggrecanase II [Cawston and Wilson, 2006; Echtermeyer *et al.*, 2009; Murphy *et al.*, 2002], our results suggest that plowing causes an increase in catabolic activities starting at 25 N applied normal force.

It is well known that cartilage has a poor intrinsic healing capacity [Lima *et al.*, 2004]. Nevertheless, after an injury, the healthy chondrocytes promote a remodeling process consisting of the elimination of the damaged matrix and in the re-building of new matrix [Treadwell *et al.*, 1991]. We could thus suppose that in the plowed cartilage, viable chondrocytes start to remodel the matrix by producing MMP-3, as shown by qRT-PCR experiments, to clear space for cell ingrowth and/or the deposition of newly synthesized proteins.

Application of the transcription inhibitor actinomycin-D before plowing reduced the MMP-3 mRNA content to its control level (Fig. 3A), indicating that early events during the 2 hours plowing period induce an increase in MMP-3 mRNA transcription. Under the same conditions, the mRNA of TIMP-1, Coll1, Agg, Fn and Coll2 were increased by a factor of two.

Assuming that in the presence of actinomycin-D the transcription was fully inhibited, as in the case of MMP-3, these results suggest that, during plowing, all these mRNAs were to a certain extent prevented from degradation and that this process is, in turn, dependent on transcription. Considering that all these mRNA content data are compared to controls not undergoing plowing, it is suggested that plowing has an additional, so far unknown, stabilizing effect on mRNAs for TIMP-1, Coll1, Agg, Fn and Coll2, which depends on active transcription. It could also be that actinomycin-D itself stabilizes certain mRNA species. The degradation of mRNA is an essential determinant in the regulation of gene expression, and it can be modulated in response to environmental signals by cis-acting elements and trans-acting factors that contribute to mRNA regulation decay [Simon *et al*., 2006; Tourriere *et al*., 2002]. Additionally, it has already been reported that actinomycin-D has a stabilizing effect on mRNA transcription of TIMP-1 by affecting trans-acting factors involved in TIMP-1 mRNA degradation [Gardner *et al*., 2006].

This stabilizing effect or artefact of actinomycin-D could also account for the 2-fold increase in Agg, Coll2, and Fn mRNA when applied for 2 hours upon plowing completion.

The 7-8-fold increase in mRNA content for TIMP-1 and Coll1, however, suggests that both species increased during plowing and in the absence of actinomycin-D decline to control levels during the 2-hours equilibration period. This hypothesis would require a post-plowing half-life of TIMP-1 and Coll1 mRNA of less than 1 hour. In contrast, the half-life of MMP-3 mRNA is around 24 hours, as deduced from figure 2. Whether or not plowing can induce factors able to modulate the half-life of specific mRNA species such as TIMP-1 or Coll1 cannot be definitively determined from these results.

In essence, we have observed three patterns of transcription regulation. The first is of the MMP-3 mRNA type, whose transcription is induced during plowing; the second includes TIMP-1 and Coll1, whose mRNA decay occurs during the early post-plowing equilibration period and the third applies to Agg, Fn and Coll2, whose basal transcription is either stabilized or unaffected by plowing.

Furthermore, we have shown that the amount of GAG release from plowed cartilage strips is higher than the corresponding release from control explants [DiMicco *et al.*, 2004; Lin *et al.*, 2004] and that this release is due to both mechanical damage and enzymatic activity. Since GAG release has also been observed after treatment of plowed cartilage with MMP inhibitor, we could state that plowing provokes mechanical degradation of the cartilage matrix. This finding is also supported by two macroscopic events, which are the bending of the strip during plowing (due to the fact that the strip is glued by the extremities to the support) and the increase in the length of the cartilage strips. The cartilage strip deformation strongly depended on the applied normal force during plowing. An increase in the entire length (Δl) of the sample was observed at all the applied forces. In more detail, for samples plowed with normal forces 25 N, 50 N and 100 N a Δl of 0.5 ± 0.07 mm; 1.0 ± 0.06 mm and 1.9 ± 0.29 mm respectively was measured. This length increase corresponded to a relative strip elongation of 0.7 ± 0.3 %, 1.5 ± 0.2 % and 3.0 ± 0.7 % for 25 N, 50 N and 100 N, respectively.

Both observations suggest that plowing could damage the collagen fibers, causing, in turn, a GAG release [DiMicco *et al.*, 2004]. Nevertheless, given that even after 1-day equilibration, the GAG release from plowed cartilage was 30% higher than in plowed cartilage treated with GM6001, we could conclude that GAG loss is also due to the catabolic activity of MMPs that are activated during plowing. Thus, the increase in MMP mRNA induced by plowing, as in the case for MMP-3, yields an increase in MMP-activity.

When cartilage explants are subjected to a static compression with an applied pressure above 0.5 MPa, the chondrocyte metabolism is already irreversibly compromised after 1 hour [Sah *et al.*, 1989; Valhmu *et al.*, 1998]. The pressure during 100 N plowing has been calculated to be around 2.5 MPa, from measurements of the contact area between the condyle and the cartilage. This finding suggests that plowing with high applied pressure is not as harmful as a static compression. In contrast to what happens during static compression, where the flow of nutrients is limited, during plowing the sliding of the indenter not only mixes the surrounding medium, thus facilitating the exchange of molecules, ensuring

358 better cartilage homeostasis, but also it squeezes fluid out of the cartilage that is
359 subsequently be replenished by new medium.
360 The response of cartilage explants to mechanical injuries has been extensively investigated
361 *in vitro* but, to our knowledge, plowing experiments where the indenter simultaneously
362 applies a compression and slides on the cartilage explants has never been reported.
363 On the basis of the obtained results, we deduce that plowing with an applied normal force of
364 100 N and an indenter speed of 10 mm/sec causes cell death of the chondrocytes closer to
365 the surface, as well as matrix damage observed as GAG loss. In addition, in healthy
366 chondrocytes, plowing promotes the production and activation of catabolic enzymes, such as
367 MMP-3 and 2 hours after plowing, shows no effect on anabolic genes such as aggrecan,
368 type-I and type II collagen, and fibronectin.

369 ## Acknowledgements

370 We are grateful to Vera Colombo and Stefan Erni for developing and installing the RPETS
371 and the tank heating system. This study was supported by the Swiss National Science
372 Foundation [SNSF, grant No. 325200-110067], by Prof. L.M. Gallo's financial start-up
373 package, and by the standard financial plan of the University of Zurich.
374
375

References

Barbosa, I., S. Garcia, V. Barbier-Chassefiere, J. P. Caruelle, I. Martelly, D. Papy-Garcia (2003) Improved and simple micro assay for sulfated glycosaminoglycans quantification in biological extracts and its use in skin and muscle tissue studies. Glycobiology *13*: 647-653.

Cawston, T. E. and A. J. Wilson (2006) Understanding the role of tissue degrading enzymes and their inhibitors in development and disease. Best Pract Res Clin Rheumatol *20*: 983-1002.

Chen, C. T., M. Bhargava, P. M. Lin, P. A. Torzilli (2003) Time, stress, and location dependent chondrocyte death and collagen damage in cyclically loaded articular cartilage. J Orthop Res *21*: 888-898.

Colombo, V., M. R. Correro, R. Riener, F. E. Weber, L. M. Gallo (2011) Design, construction and validation of a computer controlled system for functional loading of soft tissue. Med Eng Phys *33*: 677-683.

Colombo, V., M. Čadová, L. M. Gallo (2013) Mechanical behavior of bovine nasal cartilage under static and dynamic loading. J Biomech *46*: 2137–2144

Davidson, R. K., J. G. Waters, L. Kevorkian, C. Darrah, A. Cooper, S. T. Donell, I. M. Clark (2006) Expression profiling of metalloproteinases and their inhibitors in synovium and cartilage. Arthritis Res Ther *8*: R124.

DiMicco, M. A., P. Patwari, P. N. Siparsky, S. Kumar, M. A. Pratta, M. W. Lark, Y. J. Kim, A. J. Grodzinsky (2004) Mechanisms and kinetics of glycosaminoglycan release following in vitro cartilage injury. Arthritis Rheum *50*: 840-848.

Ding, L., E. Heying, N. Nicholson, N. J. Stroud, G. A. Homandberg, J. A. Buckwalter, D. Guo, J. A. Martin (2010) Mechanical impact induces cartilage degradation via mitogen activated protein kinases. Osteoarthr Cartilage *18*: 1509-1517.

Dossumbekova, A., M. Anghelina, S. Madhavan, L. He, N. Quan, T. Knobloch, S. Agarwal (2007) Biomechanical signals inhibit IKK activity to attenuate NF-kappaB transcription activity in inflamed chondrocytes. Arthritis Rheum *56*: 3284-3296.

Echtermeyer, F., J. Bertrand, R. Dreier, I. Meinecke, K. Neugebauer, M. Fuerst, Y. J. Lee, Y. W. Song, C. Herzog, G. Theilmeier, T. Pap (2009) Syndecan-4 regulates ADAMTS-5 activation and cartilage breakdown in osteoarthritis. Nat Med *15*: 1072-1076.

Fitzgerald, J. B., M. Jin, A. J. Grodzinsky (2006) Shear and compression differentially regulate clusters of functionally related temporal transcription patterns in cartilage tissue. J Biol Chem *281*: 24095-24103.

Gallo, L. M. (2005) Modeling of temporomandibular joint function using MRI and jaw-tracking technologies-mechanics. Cells Tissues Organs *180*: 54-68.

Gallo, L. M., G. Chiaravalloti, L. R. Iwasaki, J. C. Nickel, S. Palla (2006) Mechanical work during stress-field translation in the human TMJ. J Dent Res *85*: 1006-1010.

Gallo, L. M., J. C. Nickel, L. R. Iwasaki, S. Palla (2000) Stress-field translation in the healthy human temporomandibular joint. J Dent Res *79*: 1740-1746.

Gardner, J., K. Borgmann, M. S. Deshpande, A. Dhar, L. Wu, R. Persidsky, A. Ghorpade (2006) Potential mechanisms for astrocyte-TIMP-1 downregulation in chronic inflammatory diseases. J Neurosci Res *83*: 1281-1292.

Griffin, T. M. and F. Guilak (2005) The role of mechanical loading in the onset and progression of osteoarthritis. Exerc Sport Sci Rev *33*: 195-200.

Jeffrey, J. E. and R. M. Aspden (2007) Cyclooxygenase inhibition lowers prostaglandin E2 release from articular cartilage and reduces apoptosis but not proteoglycan degradation following an impact load in vitro. Arthritis Res Ther *9*: R129.

Kurz, B., A. K. Lemke, J. Fay, T. Pufe, A. J. Grodzinsky, M. Schunke (2005) Pathomechanisms of cartilage destruction by mechanical injury. Ann Anat *187*: 473-485.

Lane, S. R., M. C. Trindade, T. Ikenoue, M. Mohtai, P. Das, D. R. Carter, S. B. Goodman, D. J. Schurman (2000) Effects of shear stress on articular chondrocyte metabolism. Biorheology *37*: 95-107.

Lee, J. H., J. B. Fitzgerald, M. A. DiMicco, A. J. Grodzinsky (2005) Mechanical injury of cartilage explants causes specific time-dependent changes in chondrocyte gene expression. Arthritis Rheum *52*: 2386-2395.

Lima, E. G., R. L. Mauck, S. H. Han, S. Park, K. W. Ng, G. A. Ateshian, C. T. Hung (2004) Functional tissue engineering of chondral and osteochondral constructs. Biorheology *41*: 577-590.

Lin, P. M., C. T. C. Chen, P. A. Torzilli (2004) Increased stromelysin-1 (MMP-3), proteoglycan degradation (3B3- and 7D4) and collagen damage in cyclically load-injured articular cartilage. Osteoarth Cartilage *12*: 485-496.

Murphy, G., V. Knauper, S. Atkinson, G. Butler, W. English, M. Hutton, J. Stracke, I. Clark (2002) Matrix metalloproteinases in arthritic disease. Arthritis Res *4*(Suppl 3): S39-S49.

Palla, S., L. M. Gallo, D. Gossi (2003) Dynamic stereometry of the temporomandibular joint. Orthod Craniofac Res *6*(Suppl 1): 37-47.

Patwari, P., M. N. Cook, M. A. DiMicco, S. M. Blake, I. E. James, S. Kumar, A. A. Cole, M. W. Lark, A. J. Grodzinsky (2003) Proteoglycan degradation after injurious compression of bovine and human articular cartilage in vitro: interaction with exogenous cytokines. Arthritis Rheum *48*: 1292-1301.

Patwari, P., J. Fay, M. N. Cook, A. M. Badger, A. J. Kerin, M. W. Lark, A. J. Grodzinsky (2001) In vitro models for investigation of the effects of acute mechanical injury on cartilage. Clin Orthop Relat Res (391 Suppl): S61-S71.

Sah, R. L., Y. J. Kim, J. Y. Doong, A. J. Grodzinsky, A. H. Plaas, J. D. Sandy (1989) Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res *7*: 619-636.

Sauerland, K. and J. Steinmeyer (2007) Intermittent mechanical loading of articular cartilage explants modulates chondroitin sulfate fine structure. Osteoarthr Cartilage *15*: 1403-1409.

Sellers, W. I. and R. H. Crompton (2004) Using sensitivity analysis to validate the predictions of a biomechanical model of bite forces. Ann Anat *186*: 89-95.

Simon, E., S. Camier, B. Seraphin (2006) New insights into the control of mRNA decapping. Trends Biochem Sci *31*: 241-243.

Torzilli, P. A., M. Bhargava, S. Park, C. T. C. Chen (2010) Mechanical load inhibits IL-1 induced matrix degradation in articular cartilage. Osteoarthritis and Cartilage *18*: 97-105.

Tourriere, H., K. Chebli, J. Tazi (2002) mRNA degradation machines in eukaryotic cells. Biochimie *84*: 821-837.

Treadwell, B. V., M. Pavia, C. A. Towle, V. J. Cooley, H. J. Mankin (1991) Cartilage synthesizes the serine protease inhibitor PAI-1: support for the involvement of serine proteases in cartilage remodeling. J Orthop Res *9*: 309-316.

Valhmu, W. B., E. J. Stazzone, N. M. Bachrach, F. Saed-Nejad, S. G. Fischer, V. C. Mow, A. Ratcliffe (1998) Load-controlled compression of articular cartilage induces a transient stimulation of aggrecan gene expression. Arch Biochem Biophys *353*: 29-36.

Verteramo, A. and B. B. Seedhom (2007) Effect of a single impact loading on the structure and mechanical properties of articular cartilage. J Biomech *40*: 3580-3589.

Waldman, S. D. and J. T. Bryant (1997) Dynamic contact stress and rolling resistance model for total knee arthroplasties. J Biomech Eng *119*: 254-260.

Figure legends

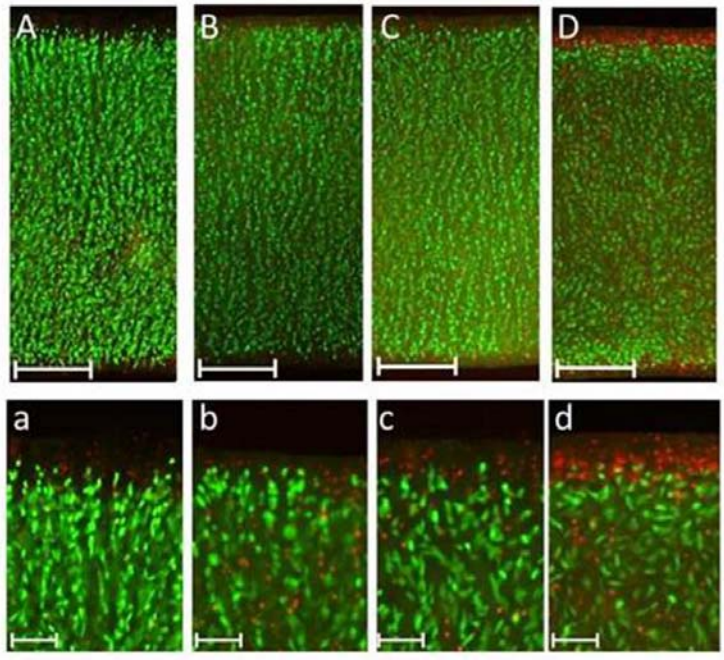

Fig.1. Chondrocyte viability after plowing at different applied normal forces. **A**, **B C and D**: Cartilage cross-sections of control cartilage and strips plowed with applied normal force of 0, 25, 50 and 100 N respectively (Scale bar = 500 μm). **a**, **b c and d** are details of A, B C and D respectively (Scale bar = 50 μm).

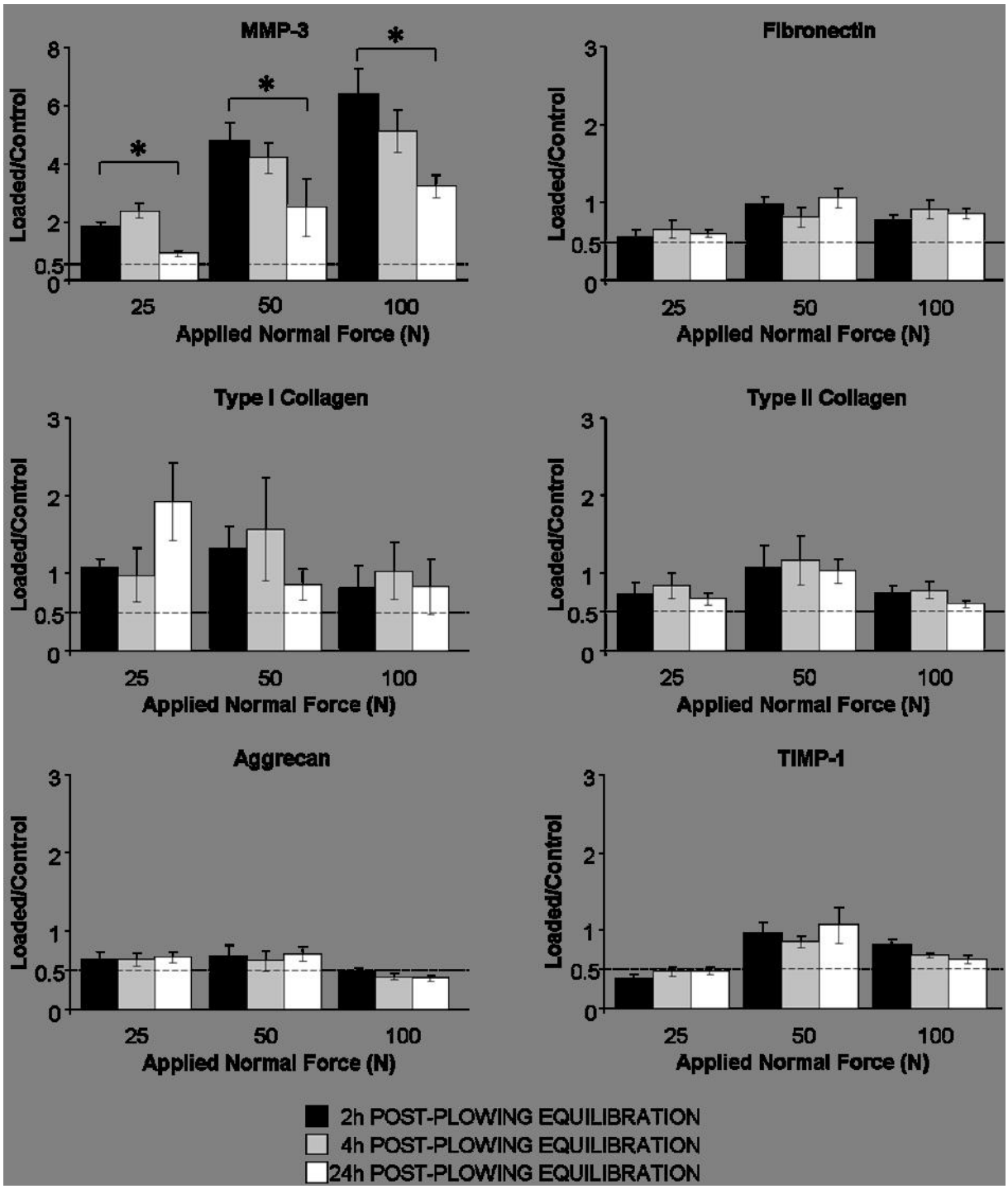

Fig.2. Gene regulation after plowing with applied normal forces 25, 50 and 100 N. Black bars: gene expression after 2h post-plowing equilibration; grey bars: gene expression after 4h post-plowing equilibration; white bars: gene expression after 24h post-plowing equilibration. Expression levels, (normalized against the G3PDH and 18S-rRNA genes), are normalized to that of controls, which were non-loaded, free-swelling explants. Each experiment was from a separate BNS (n=3), and for each experiment three, similarly treated explants were pooled for RNA extraction.

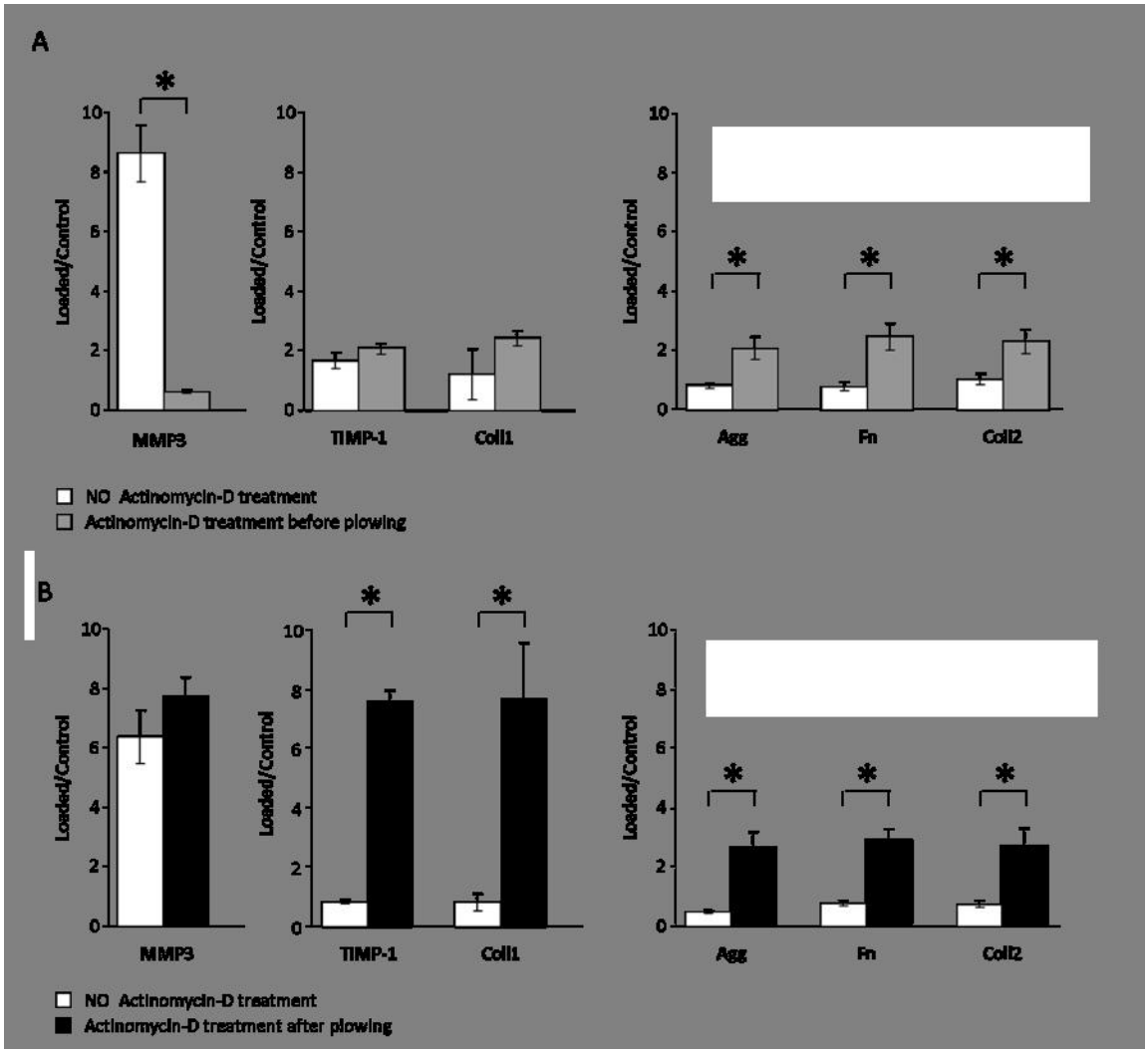

Fig.3. Gene regulation after 100 N plowing of cartilage strips not treated or treated with actinomycin-D. **A**: White bars: gene expression measured immediately after plowing with 100 N applied normal force without actinomycin-D treatment; grey bar: before plowing cartilage strips are incubated with actinomycin-D during 2 hours and the gene expression is measured after plowing. **B**: white bars: gene expression measured after 2 hours equilibration of cartilage strips plowed at 100 N applied normal force without actinomycin-D treatment; black bars: immediately after plowing, cartilage strips are incubated for 2 hours with actinomycin-D. For all experiments, the expression levels (normalized against the G3PDH and 18S-rRNA genes), are normalized to those of controls, which were non-loaded, free-swelling explants treated or not treated with actinomycin-D. Each experiment was from a separate BNS (n=3), and for each experiment three, similarly treated explants were pooled for RNA extraction.

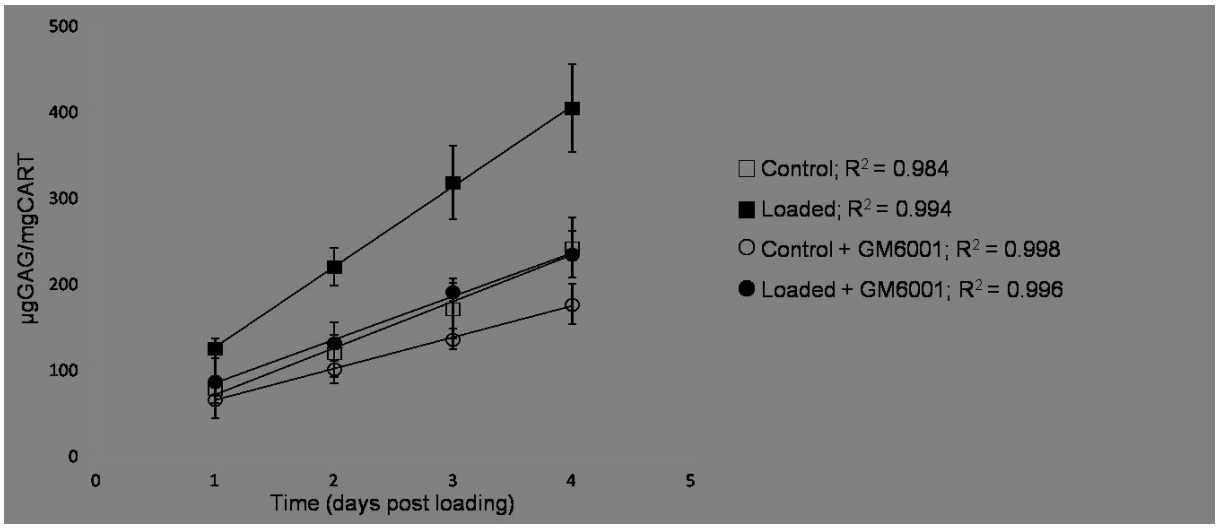

Fig.4. GAG release into the medium from 100 N plowed cartilage and control on days 1-4 post plowing (n=3 experiments for each condition and for each time point). GAG release for both loaded and control explants normalized to the corresponding cartilage wet weight. (Full squares: GAG release from plowed explants not treated with GM6001; empty squares: GAG release from control cartilage not treated with GM6001; full circles: plowed cartilage treated with GM6001; empty circles: control cartilage treated with GM6001)